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Prediction of Myotonic Dystrophy Clinical Severity Based on the Number of Intragenic $[CTG]_n$ Trinucleotide Repeats

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We carried out a genotype-phenotype correlation study, based on clinical findings in 465 patients with myotonic dystrophy (DM), in order to assess [CTG] repeat number as a predictive test of disease severity. Our analysis showed that the DM subtypes defined by strict clinical criteria fall into three different classes with a log-normal distribution. This distribution is useful in predicting the probability of specific DM phenotypes based on triplet [CTG] number. This study demonstrates that measurement of triplet expansions in patients' lymphocyte DNA is highly valuable and accurate for prognostic assessment.

KEY WORDS: myotonic dystrophy, [CTG] repeat, DNA, predictive diagnosis

INTRODUCTION

Myotonic dystrophy (DM) (MIM *160900) is an autosomal-dominant disease with an incidence of 1 in 8,000 individuals. DM is an adult-onset multisystemic disease, characterized by marked intrafamilial and interfamilial clinical variability. Patients are often divided into three groups, according to clinical symptoms and age-of-onset. The most severe form is congenital DM (CDM), which is associated with generalized muscular hypotonia, talipes, and mental retardation. The mildest form, seen in middle-to-old age, is characterized by cataracts, baldness, and minimal on absent muscle involvement. The juvenile/adult form is phenotypically variable, with myotonia, muscle weakness, cardiac arrhythmias, male balding, hypogonadism, psychocognitive dysfunction, and glucose intolerance [Harper, 1989].

Presymptomatic and prenatal testing for DM, using restriction fragment length polymorphisms (RFLPs), has been available since 1985, but its application has been limited by the need to study large pedigrees, by uninformativeness of markers, and by reduced diagnostic accuracy due to genetic recombination [Shaw et al., 1985; Meredith et al., 1986].

The cloning of the DM gene and characterization of the DM mutation at molecular level offered the possibility of direct gene testing and highly diagnosis [Harley et al., 1992a; Buxton et al., 1992; Brook et al., 1992]. The DM mutation involves an expanded [CTG] trinucleotide repeat, located at the 3′ UTR of the myotonin protein-kinase (MT-PK) gene, on the long arm of chromosome 19 [Brook et al., 1992]. The number of [CTG] tiplets in the gene varies in the normal population from 3–30. A repeat >40 [CTG] (in some patients up to a few thousands) is associated with DM [Brook et al., 1992]. The trinucleotide [CTG] is mitotically and meiotically unstable, with a bias towards length increase in the next generation, accounting for the phenomenon of "anticipation" (increasing severity in successive generations of the same family, with earlier age of onset) [Harley et al., 1992b; Harper et al., 1992]. The size of the trinucleotide repeat correlates statistically with age-of-onset and clinical severity of the disease [Harley et al., 1992b; Novelli et al., 1993; Redman et al., 1993]. Therefore, the molecular assessment of the [CTG] repeat number in the MT-PK gene might be of great help in the management of DM patients. However, the pleiotropy of this gene and the somatic instability of the DM mutation have limited the use of this

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test in predicting patients' phenotypes [Ashizawa et al., 1993].

We investigated 465 DM patients from Italy and Spain by statistical analysis of genotype-phenotype correlations, in order to test the value of the [CTG] repeat measurement as a predictor of phenotype.

MATERIALS AND METHODS

Patients

A total of 465 DM patients (267 from Italy and 198 from Spain), comprising 269 men and 196 women belonging to 215 DM families, were classified in three phenotypic classes according to clinical criteria, as reported in Table I. Clinical diagnoses and grading were performed using homogeneous criteria for all the patients. The 465 patients were examined by four different clinicians, coauthors of this paper (M.C., J.M.M., A.P., and C.A.) who were not aware of the number of CTG patients. All patients included in this study showed a DM duration of at least 10 years (class 1; range, 10–17; class 2: range, 10–32; class 3: range, 10–27). Muscular disability was defined using a five-point muscular disability rating scale (MDRS), as described by Mathieu et al. [1992]. Muscular biopsy was performed on clinically affected muscles in all patients Cryostat sections were examined by light microscopy, following routine histological and histochemical staining. Electromyography of thenar muscles was performed in all patients. Myotonia of the forearm muscles was quantified by a standardized recording procedure. Cognitive function was investigated by the MMST (mini-mental-state test) [Folstein et al., 1975]. Ocular abnormalities were detected using an ophthalmoscope and slit-lamp examination. Cardiac evaluation was done by ECC and, when indicated, by echocardiography. In addition, hospital records were available on all patients. One hundred and three patients (22%) were assigned to class 1, 205 (44%) to class 2, and 160 (34%) to class 3. There was no significant difference between Italian and Spanish patients with regard to any clinical variable tested, allowing their inclusion in the three phenotypic classes (Student's homogeneity t-test). The age of males and females was similar in each phenotypics class, ranging from 11–89 years.

DNA Analysis

All patients were studied using both polymerase chain reaction (PCR) and Southern blotting analysis, as previously described [Novelli et al., 1993]. Ten mg of lymphocyte genomic DNA was digested to completion with *Eco*RI or *Bam*HI restriction enzymes, according to the manufacturer's instructions (Stratagene, La Jolla, CA). Digestion products were electrophoresed on 0.8% agarose gels and transferred onto nylon membranes (Hybond N, Amersham, Buckinghamshire, UK). Southern blots were probed with the 1.4-Kb *Bam*HI fragment of the MDY 1 cosmid containing the variable [CTG] repeat [Fu et al., 1992]. Hybridime (HT Biotechnology, Cambridge, UK) was included at a concentration of 0.25 mg/ml in both the prehybridization and hybridization solutions. Blots were washed in 1 × sodium chloride/sodium citrate (SSC) and 0.1% sodium dodecyl sulphate (SDS) at 65°C, and then exposed to AGFA XAR film for 1–4 days. PCR was performed using 101 and 102 flanking primers, according to Brook et al. [1992]. The [CTG] repeat number was established for each patient by Southern blotting (*Eco*RI or *Bam*HI) using specific software (Image-Quant, Molecular Dynamics, Sunnyvale, CA), after densitometric analysis on a Personal Densitometer (Molecular Dynamics).

Statistical data were obtained with commercial statistics packages (SigmaStat, Jandel Scientific, Erkrath, and EXCEL 5.0, Microsoft, Redmond, WA). Multiple linear regression was used to explore the relationship between [CTG] expansion, clinical parameters, and number of analyzed subjects.

RESULTS

All patients showed an increased [CTG] repeat number in a range from 50–2,900. On average, no difference in allele size was found in patients from Italy and Spain. The normal [CTG] expansion range, determined on 200 chromosomes of unaffected control subjects, var-

TABLE I. Clinical Characteristics and Sex of the Myotonic Dystrophy Patients

Phenotypic class	Muscular disability	Other clinical symptoms	Gender
1	No muscle impairment Minimal signs of myotonia Jaw and temporal wasting Facial weakness Sternomastoids wasting or weakness Ptosis Nasal speech No distal weakness except isolated digits flexor weakness	Frontal balding Minor endocrinological signs Cataract	Male = 75 Female = 28 (P = <0.001)
2	Myotonia Distal weakness (no proximal weakness except isolated triceps brachii weakness)	EKG abnormalities Gonadal dysfunction Mild mental retardation Glucose intolerance Cataract	Male = 110 Female = 89 (P = n.s.)
3	Proximal weakness	Cardiomiopathy Endocrinological dysfunctions Mental retardation Cataract	Male = 84 Female = 79 (P = n.s.)

ied between 5–29 trinucleotides. The distribution of each allele class was superimposable to that previously described for the Italian population [Novelli et al., 1993]. The expansions showed a log-normal distribution in each class (Fig. 1). A mean of log-normal distribution of 1.98 (SD, 0.18), 2.69 (SD, 0.21), and 3.09 (SD, 0.14) was determined for phenotypic classes 1, 2, and 3, respectively. A significant sex difference was observed *only in the distribution* of class 1 (75 males vs. 28 females; $P < 0.001$).

These distributions allowed calculation of the posterior probabilities $P(C|n)$ for each patient to belong to a specific phenotypic class (C), on the basis of the [CTG] triplet number (n), using the Bayes theorem

$$P(C|n) = \frac{P(C)P(n|C)}{\sum P(C)P(n|C)}$$

where the class frequency observed in the sample was used as marginal probability $P(C)$, and the best-fit distribution function of each specific class at n as conditional probability $P(n|C)$ (Table II). Although we simply used the frequency distribution as marginal probability $P(C)$, the posterior probabilities were unchanged, as demonstrated by interval confidence values obtained on the prior probability estimates (Table II). The analysis showed that the DM subtypes defined by clinical criteria fell into three different groups, suggesting that the selection method used was highly reliable. Patients with a 100 [CTG] number have an almost 100% probability of developing a class 1 phenotype, while patients with a >1,300 [CTG] triplet number have a probability of >90% of belonging to class 3, the most severe phenotype. Although overlaps exist between the three curves, only genotypes carrying a [CTG] number of 200 or 800 triplets have a similar probability of falling into two different classes (respectively, 1 and 2, or 2 and 3).

DISCUSSION

Unstable [CTG] segregation analysis and genotype/phenotype studies have demonstrated that triplet number broadly correlates with age-at-onset and clinical severity of DM [Hunter et al., 1992; Novelli et al., 1993; Harley et al., 1993; Jaspert et al., 1995]. Furthermore, the triplet repeat length, as defined by blood DNA analysis, correlates positively with most of the individual clinical symptoms found in DM, including muscle weakness [Novelli et al., 1993; Jaspert et al., 1995], cardiac involvement (complete left bundle branch block, ventricular late potential, and decreased myocardial glucose phosphorylation) [Annane et al., 1994; Melacini et al., 1995; Tohgozoglu et al., 1995], and endocrine disorders (male hypogonadism and male infertility, and LH and FSH levels) [Mastrogiacomo et al., 1994]. The availability of a direct DNA-based test to determine the number of [CTG] repeats in the MT-PK gene is highly useful in the diagnosis of DM. This test has overcome the limits of linkage analysis, and has provided a highly sensitive and specific marker for inheritance of the mutation in affected families [Harley et al., 1992b; Novelli et al., 1993; Redman et al., 1993]. However, the lack of large prospective studies and the extreme variability of the disease have discouraged the use of blood DNA testing for prognostic assessment. In addition, the identification of biological factors, such as germline and somatic variation, gender effect, and the possible activity of modified genes, or undefined epigenetic elements which operate in the pathogenesis of the disease, have dissuaded clinicians in the past from using the repeat length for predicting DM status [Lavedan et al., 1993; Jansen et al., 1994; Wieringa, 1994; Barcelò et al., 1994; Novelli et al., 1995]. The major confounding element seems to be the frequent occurrence of gonosomal mosaicism, with different repeat expansions in different tissues of the patients [Brunner et al., 1993; Jansen et al., 1994; Massari et al., 1995]. In general, muscles show greater expansion than blood

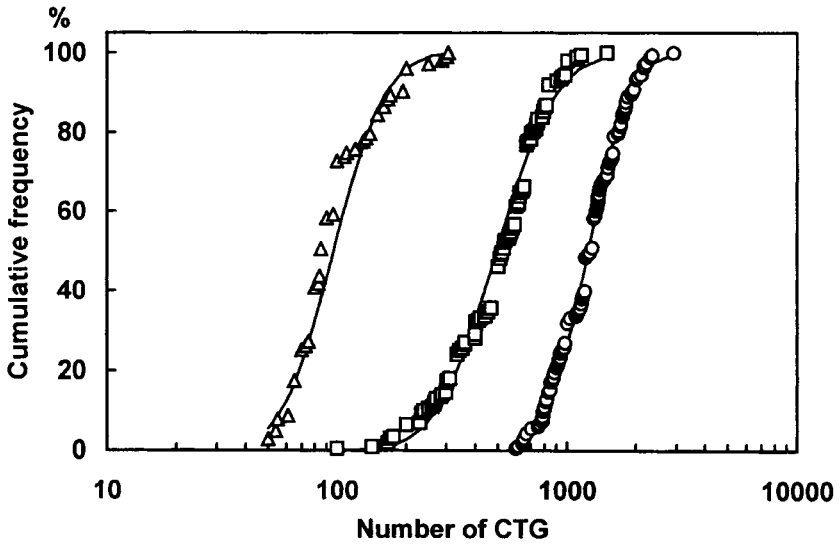

Fig. 1. Log-normal distribution function of class frequency related to [CTG] repeat number in myotonic dystrophy patients. △, class 1; □, class 2; ○, class 3.

TABLE II. Estimation of Posterior Probabilities of a Given DM Phenotype Class on the Basis of CTG Number (23 Representative Expansions)*

CTG (n)	Class 1	Class 2	Class 3
80	1.00	0.00	0.00
90	1.00	0.00	0.00
100	0.99	0.01	0.00
150	0.89 (0.02)	0.11 (0.02)	0.00
200	0.44 (0.06)	0.56 (0.04)	0.00
250	0.10 (0.01)	0.90 (0.02)	0.00
300	0.02	0.98	0.00
400	0.00	0.99	0.00
500	0.00	0.97	0.03 (0.01)
600	0.00	0.89 (0.02)	0.11 (0.02)
700	0.00	0.74 (0.03)	0.26 (0.04)
800	0.00	0.55 (0.04)	0.45 (0.05)
900	0.00	0.38 (0.04)	0.62 (0.05)
1,000	0.00	0.26 (0.03)	0.74 (0.04)
1,100	0.00	0.18 (0.03)	0.82 (0.03)
1,200	0.00	0.13 (0.02)	0.87 (0.02)
1,300	0.00	0.10 (0.02)	0.90 (0.02)
1,400	0.00	0.08 (0.01)	0.92 (0.01)
1,500	0.00	0.06 (0.01)	0.94 (0.01)
1,600	0.00	0.05 (0.01)	0.95 (0.01)
1,800	0.00	0.04 (0.01)	0.96 (0.01)
2,000	0.00	0.03 (0.01)	0.97 (0.01)
2,500	0.00	0.02	0.98

*Value variations corresponding to an interval confidence of 90% SD of prior probability estimates, in parentheses.

[Anvret et al., 1993; Massari et al., 1995], suggesting that muscle DNA analysis may be more informative and accurate than lymphocyte analysis in predicting progression of the disease [Anvret et al., 1993; Thornton et al., 1994]. However, recent studies have not disclosed any significant correlation between size of the [CTG] expansion in muscle and clinical findings, reaffirming lymphocyte DNA testing as the most sensitive and informative source for DM diagnosis [Zatz et al., 1995]. Other studies have shown a correlation between the maternal size of the [CTG] repeat in the blood and the frequency of CDM [Tsilfidis et al., 1992; Cobo et al., 1993; Ashizawa et al., 1993]. The risk of giving birth to a CDM baby is related to intergenerational amplification, and is high when the maternal DM allele is >300 repeats [Cobo et al., 1995]. One can question why blood DNA status should be more informative than other tissues for prediction of clinical outcome. It is likely that somatic expansion of the [CTG] trinucleotide is related to cell proliferation, density of nuclei, and tissue-specific factors, and that blood cells tend to retain the germline mutant [Monckton et al., 1995]. If the real predictor of clinical status is the originally inherited allele, long-term follow-up is required to improve understanding of the relationship between mutant allele and disease. However, Martorell et al. [1995] demonstrated that the repeat length in peripheral blood cells continues to enlarge throughout adult life, but it does not correlate with progression of disease. However, the observed age-dependant expansions are limited to a triplet number up to 300 repeats, frequently associated with a heterogeneous pattern on Southern blots, which renders more difficult the sizing of the repeat. In our study, only patients on whom a precise triplet measurement was detectable were included. This reduces a possible bias effect due to age-dependent somatic alteration. These results support our data and suggest that age-dependent somatic expansion does not remarkably influence the phenotype. The present survey of a representative sample of patients shows that the genomic [CTG] number is a valuable predictor of a well-defined clinical status of the disease. Genotype assessment is important both for providing more accurate informations during prenatal studies and for postnatal prognostic evaluation. In particular, testing on chorionic villi might improve the ability to predict the phenotype of an affected offspring in at-risk families. Prediction of the clinical outcome based on genotype analysis of trophoblast DNA is highly accurate, since somatic heterogeneity has not been detected in fetal tissues before 13 weeks of gestation [Wöhrle et al., 1995]. There are significantly more men that women in this study, particularly in phenotypic class 1. This could suggest that the sex of the patient is a factor contributing to clinical severity. Sex has an important role in DM transmission. In fact, segregation of the parental DM allele has some distinct influence on the triplet intergenerational changes, probably affecting postzygotic expansion of the mutation even as an evolutionary force, typical of dynamic mutations (meiotic drive) [Novelli et al., 1994; Gennarelli et al., 1994; de Munain et al., 1995]. Therefore, we suggest that the excess of males observed in class 1 is due to an ascertainment bias, probably related to the meiotic drive phenomenon, not influencing the clinical severity of the disease. This has also been confirmed by a multivariate model of analysis, in which CTG repeat number was the most significant independent predictor for neurological and cardiac abnormalities in DM patients compared to male sex, symptoms duration, and age [Tohgozoglu et al., 1995].

Predictive diagnosis of late-onset diseases is becoming increasingly feasible [Motulsky, 1994; Holtzman, 1992]. Similar testing programs have been designed for Huntington disease (HD), which is also due to the expansion of an unstable repeat. However, because of the complex nature and management of this disease, it is unlikely that HD may serve as a model for late-onset illness [Benjamin et al., 1994; Schneider et al., 1995]. On the other hand, DM is a disorder for which predictive testing can be offered to a large number of at-risk people. Guidelines for direct predictive genetic testing recommend the application of DNA analysis to those diseases where the risk prediction may be followed by treatment. Although nowadays no effective treatment is available for DM, both medical interventions and preventive measures can improve the patients' condition [Moxley, 1992]. The present limits of this test suggest its use in conjunction with extensive counselling, clinical assessment, and posttest follow-up.

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